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खाद्य विषाक्तता उत्पन्न करने वाले जीवाणुओं के संसूचन की पद्धतियां

भाग 8 कोएग्यूलेस पाज़ीटिव *स्टैफाइलोकोकाई* (*स्टैफाइलोकोकस आरियस* एवं अन्य जातियां) के गणन की समस्तर पद्धति

अनुभाग 1 बैर्ड पार्कर अगार मीडियम का उपयोग करने वाली तकनीक

Indian Standard

METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

PART 8 HORIZONTAL METHOD FOR ENUMERATION OF COAGULASE-POSITIVE *STAPHYLOCOCCI* (*STAPHYLOCOCCUS AUREUS* AND OTHER SPECIES)

Section 1 Technique Using Baird-Parker Agar Medium

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NATIONAL FOREWORD

This Indian Standard (Part 8/Sec 1) which is identical with ISO 6888-1 : 1999 'Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) — Part 1: Technique using Baird-Parker agar medium' issued by the International Organization for Standardization (ISO) was adopted by the Bureau of Indian Standards on the recommendation of the Food Microbiology Sectional Committee and approval of the Food and Agriculture Division Council.

This standard was originally published in 1970 and first revised in 1976 when it was split into parts: Part 2 covering the methods for both *staphylococci* and faecal *streptococci.* On a review by the technical committee responsible for formulating standards in this area, it was decided to revise this standard to separate the provisions and to align with the ISO Standards on the subject. Accordingly, Section 1 of Part 8 of IS 5887 covers enumeration of coagulase-positive *staphylococci* using Baird-Parker agar medium technique which is identical with ISO 6888-1 : 1999 and Section 2 of Part 8 of IS 5887 covers enumeration of coagulase-positive *staphylococci* using rabbit plasma fibrinogen agar medium technique which is identical with ISO 6888-2 : 1999. The provisions of faecal *streptococci* are retained as Part 2 of IS 5887.

In the adopted standard certain terminology and conventions are not identical to those used in Indian Standards. Attention is particularly drawn to the following:

a) Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'; and
b) Comma (,) has been used as a decimal marker while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In this adopted standard, the following International Standards are referred to. Read in their respective place, the following:

International Standard	*Corresponding Indian Standard*	*Degree of Equivalence*
ISO 6887-1 : 1999 Microbiology of food and animal feeding stuffs — Rules for the preparation of the test sample, of initial suspension and of decimal dilutions for microbiological examination — Part 1: General rules for the preparation of initial suspension and of decimal dilutions	IS 10232 : 1982 Guidelines for the preparation of dilutions for microbiological examination for food	Equivalent

The technical committee responsible for the preparation of this standard has reviewed the provisions of ISO 7218 : 1996 'Microbiology of food and animal feeding stuffs — General rules for microbiological examination' and has decided that it is acceptable for use in conjunction with this standard.

In this adopted standard, the following additional information shall be treated as added:

Clauses 5.3.1.2, 5.4	For sterilization of culture media, a temperature of 121°C for 15 minutes shall be maintained at a corresponding pressure of 103 kN/m^2 (15 psi).

For the purpose of deciding whether a particular requirement of this standard is complied with the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS 2 : 1960 'Rules for rounding of numerical values (*revised*)'. The number of significant places retained in the rounded off value, should be the same as that of the specified value in this standard.

Indian Standard
METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

PART 8 HORIZONTAL METHOD FOR ENUMERATION OF COAGULASE-POSITIVE *STAPHYLOCOCCI* (*STAPHYLOCOCCUS AUREUS* AND OTHER SPECIES)

Section 1 Technique Using Baird-Parker Agar Medium

1 Scope

This part of ISO 6888 specifies a horizontal method for the enumeration of coagulase-positive staphylococci in products intended for human consumption or feeding of animals, by counting of colonies obtained on a solid medium (Baird-Parker medium) after aerobic incubation at 35 °C or 37 °C.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this part of ISO 6888. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 6888 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Rules for the preparation of the test sample, of initial suspension and of decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and of decimal dilutions.*

ISO 7218, *Microbiology of food and animal feeding stuffs — General rules for microbiological examination.*

3 Terms and definitions

For the purposes of this part of ISO 6888, the following terms and definitions apply.

3.1
coagulase-positive staphylococci
bacteria which form typical and/or atypical colonies on the surface of a selective culture medium and which show a positive coagulase reaction when the test is performed following the method specified in this part of ISO 6888

3.2
enumeration of the coagulase-positive staphylococci
determination of the number of coagulase-postive staphylococci found per millilitre or per gram of sample when the test is carried out according to the method specified in this part of ISO 6888

4 Principle

4.1 Inoculation of the surface of a solid selective culture medium, using duplicate plates, with a specified quantity of the test sample if the product is liquid, or with a specified quantity of the initial suspension in the case of other products.

Inoculation, under the same conditions, using decimal dilutions of the test sample or of the initial suspension, with two plates per dilution.

4.2 Aerobic incubation of the plates at 35 °C or 37 °C[1] and examination after both 24 h and 48 h.

4.3 Calculation of the number of coagulase-positive staphylococci per millilitre, or per gram, of sample from the number of typical and/or atypical colonies obtained on plates at dilution levels chosen so as to give a significant result, and confirmed by a positive coagulase test result.

5 Diluent and culture media

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Diluent

See ISO 6887-1 and the specific standard dealing with the product to be examined.

5.3 Baird-Parker agar medium[2]

NOTE Commercially available media may be used. In such cases, the manufacturer's instructions should be followed carefully.

5.3.1 Base medium

5.3.1.1 Composition

Pancreatic digest of casein	10,0 g
Yeast extract	1,0 g
Meat extract	5,0 g
Sodium pyruvate	10,0 g
L-Glycine	12,0 g
Lithium chloride	5,0 g
Agar	12 g to 22 g 1)
Water, to a final volume of	1 000 ml
1) Depending on the gel strength of the agar.	

5.3.1.2 Preparation

Dissolve the components or the dehydrated complete base in the water by boiling.

If necessary, adjust the pH so that after sterilization it is 7,2 ± 0,2 at 25 °C.

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1) The temperature is agreed between the interested parties and is indicated in the test report.

2) The agar medium is that of Baird-Parker (see reference [3]) with the addition of sulfamezathine (see reference [4]) if the presence of *Proteus* is suspected.

Transfer the medium in quantities of 100 ml to flasks or bottles (6.5) of appropriate capacity.

Sterilize the medium for 15 min at 121 °C.

5.3.2 Solutions

5.3.2.1 Potassium tellurite solution

5.3.2.1.1 Composition

Potassium tellurite [1] (K_2TeO_3)	1,0 g
Water	100 ml

[1] It is recommended to ensure beforehand that the potassium tellurite available is suitable for this test (see 5.3.2.1.2).

5.3.2.1.2 Preparation

Dissolve the potassium tellurite completely in the water with minimal heating.

The solid should be readily soluble. If a white insoluble material is present in the water, discard the powder.

Sterilize by filtration using 0,22 µm pore size membranes.

The solution may be stored at the maximum for one month at +3 °C ± 2 °C.

Discard the solution if a white precipitate forms.

5.3.2.2 Egg yolk emulsion (concentration approximately 20 % or according to the manufacturer's instructions)

NOTE If a commercial preparation is available, it should be used.

Use fresh hen eggs with intact shells. Clean the eggs with a brush using a liquid detergent. Rinse them under running water, then disinfect the shells either by immersing them in ethanol (70 % volume fraction) for 30 s and allowing them to dry in the air, or by spraying them with alcohol followed by flame sterilization.

Proceeding under aseptic conditions, break each egg and separate the yolk from its white by repeated transfer of the yolk from one half of the shell to the other. Place the yolks in a sterile flask (6.5) and add four times their volume of sterile water. Mix thoroughly. Heat the mixture in the water bath (6.4) set at 47 °C for 2 h and leave for 18 h to 24 h at +3 °C ± 2 °C to allow a precipitate to form. Aseptically collect the supernatant liquid into a fresh sterile flask for use.

The emulsion may be stored at +3 °C ± 2 °C for a maximum of 72 h.

5.3.2.3 Sulfamezathine (sulfamethazine, sulfadimidine) solution

NOTE This is to be used only if *Proteus* species are suspected in the test sample.

5.3.2.3.1 Composition

Sulfamezathine	0,2 g
Sodium hydroxide solution, $c(\text{NaOH}) = 0,1$ mol/l	10 ml
Water	90 ml

5.3.2.3.2 Preparation

Dissolve the sulfamezathine in the sodium hydroxide solution.

Dilute to 100 ml with the water.

Sterilize by filtration using 0,22 µm pore size membranes.

The solution may be stored at the maximum for one month at +3 °C ± 2 °C.

5.3.3 Complete medium

5.3.3.1 Composition

Base medium (5.3.1)	100 ml
Potassium tellurite solution (5.3.2.1)	1,0 ml
Egg-yolk emulsion (5.3.2.2)	5,0 ml
Sulfamezathine solution (5.3.2.3) (if necessary)	2,5 ml

5.3.3.2 Preparation

Melt the base medium, then cool it to approximately 47 °C by means of the water bath (6.4).

Add, under aseptic conditons, the two other solutions (5.3.2.1 and 5.3.2.2) and if necessary (if *Proteus* species are suspected in the test sample) the sulfamezathine solution (5.3.2.3), each solution being previously warmed in a water bath at 47 °C, mixing well after each addition.

5.3.4 Preparation of agar plates

Place the appropriate quantity of the complete medium (5.3.3) into sterile Petri dishes in order to obtain an agar thickness of about 4 mm, and allow to solidify.

The plates may be stored, prior to drying, for up to 24 h at +3 °C ± 2 °C.

NOTE The manufacturer's instructions should be followed concerning the storage period for industrially prepared plates.

Before use, dry the plates, preferably with the lids off and the agar surface downwards, in an oven set at a temperature between 25 °C and 50 °C, until the droplets have disappeared from the surface of the medium.

5.4 Brain-heart infusion broth

5.4.1 Composition

Enzymatic digest of animal tissues	10,0 g
Dehydrated calf brain infusion	12,5 g
Dehydrated beef heart infusion	5,0 g
Glucose	2,0 g
Sodium chloride	5,0 g
Disodium hydrogenphosphate, anhydrous (Na_2HPO_4)	2,5 g
Water	1 000 ml

5.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, heating if necessary.

Adjust the pH so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Transfer the culture medium in quantities of 5 ml to 10 ml to tubes or bottles (6.5) of appropriate capacity.

Sterilize the medium for 15 min at 121 °C.

5.5 Rabbit plasma

Use commercially available dehydrated rabbit plasma and rehydrate it according to the manufacturer's instructions.

If dehydrated rabbit plasma is not available, dilute one volume of fresh sterile rabbit plasma with three volumes of sterile water.

Add EDTA (ethylenediaminetetraacetic acid) solution to give 0,1 % EDTA in the rehydrated or diluted plasma, if potassium citrate or sodium citrate has been used as the plasma anticoagulant [3].

Unless stated by the manufacturer, the rehydrated or diluted plasma shall be used immediately.

Before use, test each batch of plasma with coagulase-positive strains of staphylococci and strains of coagulase-negative staphylococci.

6 Apparatus and glassware

NOTE Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) and **wet sterilization (autoclave)**

See ISO 7218.

6.2 Incubator, for maintaining the inoculated media, plates and tubes within the temperature range 35 °C ± 1 °C or 37 °C ± 1 °C.

6.3 Drying cabinet or **incubator**, capable of being maintained at between 25 °C ± 1 °C and 50 °C ± 1 °C.

6.4 Water bath, or similiar apparatus, capable of being maintained at 47 °C ± 2 °C.

6.5 Test tubes, flasks or **bottles with screw caps**, of appropriate capacity, for sterilization and storage of culture media and incubation of liquid media; in particular, sterile haemolysis tubes, or round-bottom bottles of approximate dimensions 10 mm × 75 mm.

6.6 Petri dishes, sterile, made of glass or plastic.

6.7 Straight wire (see ISO 7218) and **Pasteur pipette**.

6.8 Total-delivery graduated pipettes, of nominal capacities 1 ml, 2 ml and 10 ml, graduated in 0,1 ml, 0,1 ml and 0,5 ml divisions, respectively.

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[3] Oxalated or heparinized plasma does not require EDTA (see reference [5]).

6.9 Spreaders, sterile, made of glass or plastic.

6.10 pH-meter, capable of being read to the nearest 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to ± 0,1 pH unit.

7 Sampling

Sampling is not part of the method specified in this part of ISO 6888. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887-1 and the specific standard appropriate to the product concerned.

9.2 Inoculation

9.2.1 Transfer, by means of a sterile pipette (6.8), 0,1 ml of the test sample if liquid, or 0,1 ml of the initial suspension (10^{-1} dilution) in the case of other products, to each of two agar plates (5.3.4). Repeat the procedure for the 10^{-2} dilution and for further decimal dilutions if necessary.

9.2.2 If, for certain products, it is desirable to count low numbers of coagulase-positive staphylococci, the limits of detection can be raised by a factor of 10 by inoculating 1,0 ml of the test sample if liquid, or 1,0 ml of the initial suspension for other products, either on the surface of one large agar plate (140 mm) or on the surface of three small agar plates (90 mm). In both cases, prepare duplicates by using two large plates or six small ones.

9.2.3 Carefully spread the inoculum as quickly as possible over the surface of the agar plate, trying not to touch the sides of the dish, using the spreader (6.9). Allow the plates to dry with their lids on for about 15 min at laboratory temperature.

9.3 Incubation

Invert the plates prepared according to 9.2.3 and incubate them for 24 h ± 2 h then re-incubate for a further 24 h ± 2 h in the incubator (6.2) at 35 °C or 37 °C[4].

9.4 Selection of plates and interpretation

9.4.1 After incubation for 24 h ± 2 h, mark on the bottom of the plates the positions of any typical colonies present (see note 1).

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4) The temperature is agreed between the interested parties and is indicated in the test report.

Re-incubate all plates at 35 °C or 37 °C[5] for a further 24 h ± 2 h, and mark any new typical colonies. Also mark any atypical colonies present (see note 1).

Take for enumeration only those plates (see note 2) that contain at the maximum 300 colonies with 150 typical and/or atypical colonies at two successive dilutions. One of the plates shall contain at least 15 colonies. Select for confirmation (9.5) a given number A (in general 5 typical colonies if there are only typical colonies, or 5 atypical colonies if there are only atypical colonies, or 5 typical colonies and 5 atypical colonies if both types are present, from each plate).

If there are less than 15 typical and/or atypical colonies present on plates inoculated with undiluted liquid product or the lowest dilution of other products, it is possible to make an estimated count as described in 9.4.3 and 10.2.

NOTE 1 Typical colonies are black or grey, shining and convex (1 mm to 1,5 mm in diameter after incubation for 24 h and 1,5 mm to 2,5 mm in diameter after incubation for 48 h) and surrounded by a clear zone. After incubation for at least 24 h an opalescent ring, immediately in contact with the colonies, may appear in this clear zone.

Atypical colonies may present one of the following morphologies:

a) shining black colonies with or without a narrow white edge; the clear zone is absent or barely visible and the opalescent ring is absent or hardly visible;

b) grey colonies free of clear zones.

Atypical colonies are formed mainly by strains of coagulase-positive staphylococci contaminating, for example dairy products, shrimps and giblets. They are less often formed by strains of coagulase-positive staphylococci contaminating other products.

NOTE 2 Bacteria belonging to genera other than staphylococci may give colonies with an appearance similar to staphylococci. Microscopic examination of Gram stain, before confirmation, will enable the distinction of other genera from staphylococci.

9.4.2 If a 1,0 ml inoculum was spread over three plates (see 9.2.2), treat these plates as one in all subsequent counting and confirmation procedures.

9.4.3 To make an estimated count of lower numbers of coagulase-positive staphylococci, retain all plates that contain any typical and atypical colonies. Select all such colonies for confirmation within the limits set out above.

9.5 Confirmation (coagulase test)

From the surface of each selected colony (9.4), remove an inoculum with a sterile wire (6.7) and transfer it to a tube or bottle of brain-heart infusion broth (5.4).

Incubate at 35 °C or 37 °C[5] for 24 h ± 2 h.

Aseptically add 0,1 ml of each culture to 0,3 ml of the rabbit plasma (5.5) (unless other amounts are specified by the manufacturer) in sterile haemolysis tubes or bottles (specified in 6.5), and incubate at 35 °C or 37 °C[5].

By tilting the tube, examine for clotting of the plasma after 4 h to 6 h of incubation and, if the test is negative, re-examine at 24 h of incubation, or examine at the incubation times specified by the manufacturer.

Consider the coagulase test to be positive if the volume of clot occupies more than half of the original volume of the liquid.

As a negative control, for each batch of plasma, add 0,1 ml of sterile brain-heart infusion broth (5.4) to the recommended quantity of rabbit plasma (5.5) and incubate without inoculation. For the test to be valid, the control plasma shall show no signs of clotting.

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[5] The temperature is agreed between the interested parties and is indicated in the test report.

10 Expression of results

10.1 General case

10.1.1 Calculation of the number a of coagulase-positive staphylococci identified for each plate selected

Calculate, for each of the plates, the number a of identified coagulase-positive staphylococci, according to the equation:

$$a = \frac{b_c}{A_c} \times c_c + \frac{b_{nc}}{A_{nc}} \times c_{nc}$$

where

A_c is the number of typical colonies submitted to the coagulase test (9.5);

A_{nc} is the number of atypical colonies submitted to the coagulase test (9.5);

b_c is the number of typical colonies which have been shown to be coagulase-positive;

b_{nc} is the number of atypical colonies which have been shown to be coagulase-positive;

c_c is the total number of typical colonies seen on the plate (9.4);

c_{nc} is the total number of atypical colonies seen on the plate (9.4).

Round off to a whole number (see ISO 7218).

10.1.2 Calculation of the number N of identified coagulase-positive staphylococci present in the test portion

For those dishes containing at the maximum 300 colonies, with 150 typical and/or atypical colonies at two consecutive dilutions, calculate the number of coagulase-positive staphylococci for each dish as specified in 10.1.1 and calculate, as a weighted mean from the two successive dilutions, the number N of identified coagulase-positive staphylococci present in the test sample, using the following equation:

$$N = \frac{\sum a}{V(n_1 + 0{,}1\, n_2)d}$$

where

$\sum a$ is the sum of the coagulase-positive staphylococcal colonies identified on all the dishes selected;

V is the volume of inoculum on each dish, in millilitres;

n_1 is the number of dishes selected at the first dilution;

n_2 is the number of dishes selected at the second dilution;

d is the dilution rate corresponding to the first dilution selected (the initial suspension is a dilution).

Round off the calculated results to two significant figures (see ISO 7218).

Report the result as the number of coagulase-positive staphylococci per millilitre (liquid products) or per gram (other products), expressed as a number between 1,0 and 9,9 inclusive multiplied by 10^x where x is the appropriate power of 10.

10.1.3 Example

A count of a product after inoculation with 0,1 ml of product gave the following results:

— for the first dilution selected (10^{-2}): 65 typical colonies and 85 typical colonies and no atypical colonies;

— for the second dilution selected (10^{-3}): 3 typical colonies and 7 typical colonies and no atypical colonies.

The following numbers were stabbed:

— from 65 colonies, 5 colonies were stabbed and all 5 proved to be coagulase positive, giving $a = 65$;

— from 85 colonies, 5 colonies were stabbed, 3 of which proved to be coagulase positive, giving $a = 51$;

— from 3 colonies, all 3 were stabbed and proved to be coagulase positive, giving $a = 3$;

— from 7 colonies, 5 colonies were stabbed and all 5 proved to be coagulase positive, giving $a = 7$.

$$N = \frac{65 + 51 + 3 + 7}{0{,}22 \times 10^{-2}} = 57\,272$$

The result, after rounding off, is $5{,}7 \times 10^4$.

10.2 Estimation of low numbers

10.2.1 If the two dishes, corresponding to the test sample (liquid products) or the initial suspension (other products) each contain less than 15 identified colonies, report the result as follows.

a) For liquid products, estimated number of coagulase-positive staphylococci per millilitre:

$$N_e = \frac{\sum a}{V \times 2}$$

where

$\sum a$ is the sum of the colonies of coagulase-positive staphylococci identified (10.1.1) on the two dishes selected;

V is the volume spread over each dish.

b) For other products, estimated number of coagulase-positive staphylococci per gram:

$$N_e = \frac{\sum a}{V \times 2 \times d}$$

where

$\sum a$ is the sum of the colonies of coagulase-positive staphylococci identified (10.1.1) on the two dishes selected;

d is the dilution rate of the initial suspension;

V is the volume spread over each dish.

10.2.2 If the two dishes, corresponding to the test sample (liquid products) or the initial suspension (other products) do not contain any colonies of coagulase-positive staphylococci and if the inoculation has been performed with 0,1 ml of sample, report the result as follows (general case of a 0,1 ml inoculum):

— less than 10 coagulase-positive staphylococci per millilitre (liquid products);

— less than $10/d$ coagulase-positive staphylococci per gram (other products), where d is the dilution rate of the initial suspension.

If the inoculation has been performed with 1 ml of sample, report the result as follows:

- less than 1 coagulase-positive staphylococcus per millilitre (liquid products);
- less than $1/d$ coagulase-positive staphylococcus per gram (other products).

11 Precision

See ISO 7218.

12 Test report

The test report shall specify:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, with reference to this part of ISO 6888;
- the incubation temperature used;
- all operating details not specified in this part of ISO 6888, or regarded as optional, together with details of any incidents which may have influenced the test results;
- the results obtained.

Bibliography

[1] ISO 6888-2, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) — Part 2: Technique using rabbit plasma fibrinogen agar medium.*

[2] KLOOS W.E. Systematics and the natural history of staphylococci. In: Staphylococci, *J. Appl. Bacteriol. Symp. Suppl.*, **69**, 1990, pp. 25 s - 37 s; and *Bergey's Manual of Determinative Bacteriology*, 9th edn., 1994.

[3] BAIRD-PARKER, A.C. J. Appl. Bacteriol., **25** (1), 1962, pp. 12-19.

[4] SMITH, B.A. and BAIRD-PARKER, A.C. J. *Appl. Bacteriol.,* **27** (1), 1964, pp. 78-82.

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Northern	: SCO 335-336, Sector 34-A, CHANDIGARH 160 022	60 38 43 60 20 25
Southern	: C.I.T. Campus, IV Cross Road, CHENNAI 600 113	254 12 16, 254 14 42 254 25 19, 254 13 15
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